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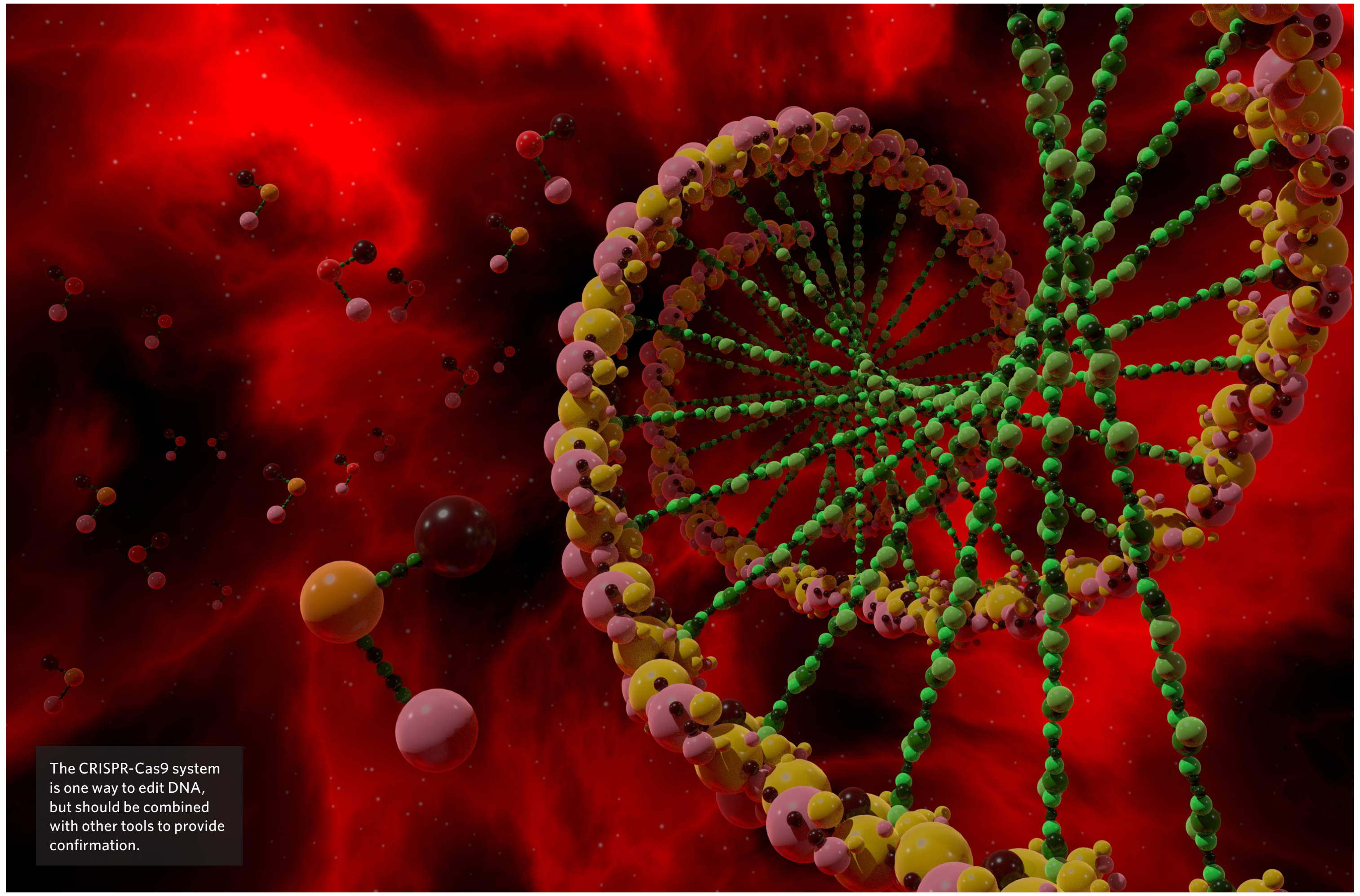
PARALLEL LINES: WHY ORTHOGONAL VALIDATION STRENGTHENS GENE-MODULATION RESEARCH

From RNA interference to CRISPR, researchers have **SEVERAL POWERFUL METHODS AT THEIR FINGERTIPS TO MANIPULATE GENE FUNCTION.** Used synergistically, such techniques make genetic perturbation studies more robust.



The CRISPR-Cas9 system is one way to edit DNA, but should be combined with other tools to provide confirmation.

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Scientists at Cold Spring Harbor (CSH) Laboratory in Long Island, New York, believed without doubt that a protein called MELK was vital for cancer growth. After all, dozens of papers had confirmed that this molecule (which is found in high levels in tumours) helped cancer cells thrive. Several promising MELK inhibitors were already in clinical trials. But when the CSH team, led by geneticist, Jason Sheltzer, used gene-editing technique CRISPR to turn off the MELK gene in a cancer cell line, they were astounded to find that absolutely nothing happened.[1] The tumour cells continued to divide, unfazed by the genetic robbery. "For weeks, I thought the results looked different because I set up the experiments incorrectly," says first author, Ann Lin, then an undergraduate in Sheltzer's lab. But time and again, she got the same findings.

That surprising discovery in 2017 led the scientists to wonder: had other drugs' targets been mischaracterized too? Sure enough, follow-up research found at least 10 oncology drug candidates that did not have the mechanism of action experts thought they did.[2] The potential therapies were still able to kill the cancer cells when the target protein was knocked out with CRISPR. *In vitro*, the medicines were working, just not the way they were supposed to.

Before CRISPR, another gene-editing technique dominated. RNA interference (RNAi) revolutionized gene function experiments by allowing scientists to use small pieces of RNA to shut down the translation of genes to proteins. RNAi remains a simple yet powerful technique, but it has limitations depending on the aim of the experiment. It reduces gene expression significantly, but doesn't switch the gene off entirely. And off-target effects can sometimes occur, where silencing the translation of one gene has an unintended impact on other proteins.

This is what the CSH researchers believe happened with the anti-MELK molecules: other genes were responsible for the cancer-killing powers. And the story highlights the importance of orthogonal validation—combining different experimental techniques to ensure gene function studies pass the reproducibility test, or to quickly and efficiently discover whether you're chasing an experimental ghost. "By using complementary approaches, researchers can minimize the likelihood that one technique's shortcomings lead to a false finding," sums up Lin.

It's a knockout

All gene-editing techniques have their strengths and weaknesses. RNAi reduces gene expression at the messenger RNA level. CRISPR, which employs a short strand of RNA attached to a DNA-cleaving enzyme (typically Cas9), instead permanently silences the gene at the DNA level. A CRISPR knockout (CRISPRko), therefore, allows researchers to be sure that the resulting phenotype is directly related to the complete silencing of the gene.

With the target gene entirely absent, scientists can test their hypothesis to see if cells respond in the way they expect them to. "CRISPR has brought this ability to highly target genes of interest at any scale," says Ryan Donnelly, senior product manager at cell-engineering company Horizon Discovery. "Knocking out gene function at the DNA level has really opened up and simplified target identification."

That said, a CRISPRko screen also has its challenges. "Some genes are essential to cell life," explains Donnelly. Completely knocking out the function of one of them kills the cells, and scientists lose the ability to interrogate the gene further.[3] They would know the gene is vital for cell survival, but not necessarily why. It is preferable, instead, to vary the gene's expression and see how that changes the cells' phenotype.

But there are other ways of using CRISPR that don't completely snuff out the gene of interest. Researchers can use a deactivated enzyme to inhibit gene expression without permanently changing the DNA.[4] This technique, known as CRISPR-interference (CRISPRi) allows targeted 'knockdown', rather than knockout. "CRISPRi represses the gene of interest, instead of turning it off," Donnelly explains. "I think of it as more of a dimmer switch than a light switch." You can also turn the dimmer up with a technique called CRISPR-activation (CRISPRa). This method leads to overexpression of the specific gene, revealing other aspects of its role in the phenotype.

The full complement

That's not to dismiss knockdown pioneer RNAi. It remains the simplest way to mute a gene of interest. "RNAi allows for reversible gene silencing, unlike most applications of CRISPR/Cas9 where edits to the genome are permanent," says Lin. Plus, there are ways to mediate off-target effects using antisense seed-region chemical modifications, which Horizon uses in its premium siRNA platform.

For many research projects, Donnelly says it makes sense to start with RNAi, then follow it with another technique, such as CRISPRko or CRISPRi, to confirm the phenotype. Or conduct complementary CRISPRi and CRISPRa experiments to find genes that are critical to both inhibition and activation of a specific biochemical pathway. "It's not that one method is wrong," says Donnelly. "It's about using other tools to improve your confidence." Another approach is to apply the same techniques to a more biologically relevant cell type, such as a primary cell model, in a follow-up experiment, to see if it gives the same results, he suggests.

Using the full suite of gene-manipulation tools can prevent scientists from jumping to wrong conclusions, says Donnelly. Combining the powers of two or more methods provides a fuller picture. That extra clarity is invaluable for researchers looking to better understand a drug's mechanism of action or find a reliable therapeutic target. And showing that the phenotypic effects of a target are reproducible across a variety of experimental models can help get your work published in higher-tier journals, he adds.

Of course, eliminating a target is often just as important as confirming one. For instance, if CRISPRi and RNAi don't align, it could be that the hit you thought you'd identified isn't really there. The silver lining, says Donnelly, is that you're weeding out the targets that won't make the grade as early as possible, giving you more confidence in the hits you do get. Firms can save their investment for the candidates more likely to progress. "It does take more effort, time and expense to generate these stronger datasets," says Donnelly. "But it's better to invest that time upfront than to have weaker targets move their way through trials. That gets very expensive very quickly." ■

REFERENCES

1. Lin, A. *et al.* eLife 6 e24179. (2017).
2. Lin, A. *et al. Science translational medicine* **11**,509: eaaw8412 (2019).
3. Wang, B., *et al. J Biol Eng* **13**, 35 (2019).
4. Gilbert, L.A. *et al. Cell* **154**,2: 442-51 (2013).

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